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Most cells have four fundamental goals; they must replicate their DNA, segregate their chromosomes, double their mass, and divide. Coordination of these events is essential. I have used the budding yeast *S. cerevisiae* to dissect the molecular events that control cell cycle progression. I have shown that not only are G1 cyclin-CDK complexes highly unstable rate-limiting activators for cell cycle progression, but that their essential function is to regulate the initiation of DNA replication by phosphorylating and promoting the degradation of a key cell cycle inhibitor, Sic1 (1,2). In fact, cyclin-CDK-regulated proteolysis may constitute the major mechanism of cell cycle control (3). Moreover, Sic1 appears to be the analog of the RB tumor suppressor gene. I have shown that Sic1, like RB, controls the timing of S-phase, ensures genomic stability, and most significantly, *sic1* deletions, like *rb* deletions, completely remove cell cycle dependence on G1 cyclins (1,22). Furthermore, Sic1 protein levels may set the G1 cyclin threshold for cell cycle progression. Ongoing research focuses on two questions: 1) How does Sic1 or other key substrate(s) control cell cycle progression? and 2) What mechanism couples and coordinates cell growth with cell cycle progression?

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Introduction

Cell cycle regulation is a fundamental biological function and the basic mechanisms are highly conserved throughout evolution. In eukaryotes, cell cycle progression appears to be precisely orchestrated by a cascade of tightly regulated cyclin-dependent kinases (4). Implicit in this observation is the hypothesis that cyclin-dependent phosphorylations drive the cell cycle. However, despite the fact that this fundamental mechanism is conserved from yeast to humans, we as yet, know very little of molecular details of this process. There is a clear need to uncover the key targets of cyclin-dependent kinases, and to learn how the phosphorylation of these substrates promotes cell division.

Cell division in yeast and humans is regulated at G1/S boundary at a "point" known as Start in yeast or the "restriction point" in mammalian cells (5-8). This "point" has been functionally defined as the moment in time at which a cell becomes irreversibly committed to cell cycle progression. In mammalian cells, this "point" has been experimentally defined as the time at which cell cycle progression becomes insensitive to serum withdrawal (9). Phosphorylation of the Retinoblastoma protein by cyclin D and cyclin E Cdk2 complexes is highly correlative with passage of cells past the "restriction point. (9). Similarly, it is well established in yeast that Start is completely dependent upon an active cyclin dependent Cdc28 kinase complex (4, 10, 11). In both yeast and nontransformed human cells, commitment to cell cycle progression is intrinsically dependent upon growth factor signaling pathways and a critical rate of protein synthesis (9). Loss of adequate nutritional status, or a precipitous drop in protein synthesis rates results in exit from the cell cycle (9). Substantial experimental research has indicated that cells must accumulate a critical threshold of a highly labile protein, the R protein, before they can pass the restriction point/Start (9). As shown below, I provide strong evidence that this activity is in fact due to G1 cyclins. Remarkably cancer cells appear to be completely resistant to this form of cell cycle control. The significance of this observation is discussed in light of aforementioned evidence.

A link between cell cycle control and cancer has long been suspected, but only recently have the two fields completely merged. This is strikingly evident for breast cancers in particular. Fifty percent of adenocarcinomas of the breast show 11q13 chromosomal amplifications (12). This led to the cloning of PRAD1/Cyclin D1 as the first clearly oncogenic human cell cycle gene (13). Overexpression of human cyclin D1 or E is not only rate limiting for G1 progression in breast cancers, but also an excellent molecular marker for distinguishing malignant and invasive breast carcinomas from non-malignant lesions (14,15).

It is generally accepted that overexpression of G1 cyclins leads to inappropriate activation of cyclin-dependent kinases (16). However, despite the fact that a large number of cyclin dependent kinases have been discovered, we know very little about how they function to promote cell cycle progression. Specifically, there is a clear paucity of known substrates, and no clear mechanism whereby phosphorylation of key substrates explains critical cell cycle events. Two questions need to be answered: What are the relevant in vivo cyclin dependent kinase substrates for cell cycle progression? and What effect does phosphorylation have on these substrates? The ease of its propagation, its completely sequenced genome, and its tractability to genetic manipulation make the yeast *S. cerevisiae* an excellent experimental organism in which to explore these basic questions.

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In the budding yeast *S. cerevisiae*, G1 cells increase in mass until they achieve a critical cell size. At critical cell size, Start occurs which commits cells to a full round of division. Prior to the beginning of this project, Start as an event was poorly understood and remained operationally defined as an intangible irreversible point in cell cycle progression (5, 8, 11). In part due to the research described here, we know believe that Start is in fact the coupling and coordination of many separable cell cycle events (e.g. the initiation of DNA synthesis, the duplication of spindle pole bodies, and the initiation of budding). Biochemical and genetic studies suggested that Start is regulated by a G1 cyclin dependent kinase Cdc28.

Another important molecule is Sic1. It was identified as a co-precipitated substrate of Cdc28 (17). Although it has no homology to any other Cdk inhibitors, Sic1 is a potent and specific inhibitor of all cyclin B-type-Cdc28 kinase complexes (18-21). I have recently identified a Sic1 as not only a putative Cdk substrate, but also an important molecule in controlling cell cycle progression.

This project investigates how specific genes regulate Start. The work in this report addresses two questions: 1) What mechanism couples and coordinates cell growth with cell cycle progression? and 2) Are the Clns the functional equivalent of the highly labile R-protein(s) in mammalian cells?

Body

The questions addressed above are discussed in the following two sections. In section (A), I briefly summarize how each of the technical objectives in the Statement of Work has been addressed in the last year. This work will be submitted as a manuscript by the end of the year. As a direct result of the work proposed here, I was able to pursue a related line of research in collaboration with three other yeast laboratories: (Tyers, Mendenhall, and Wittenberg labs). This work, which is in press at Nature, is presented in section (B) and demonstrates that G1 cyclins (Clns) in yeast are highly unstable activators of cell cycle progression. We conclude that Cln protein stability is not cell cycle regulated, that G2 cyclins are not required for Cln instability, and that constituitive Cln instability is essential for the proper coordination of cell growth and cell division. These traits make Clns ideal candidates for highly labile R-proteins described in both yeast and humans.

Size Control, Sic1, and "Cln thresholds"

As laid out in my in my Statement of work, this section addresses the two technical objectives that I set out to pursue during the first year of this grant: 1) Characterization of Sic1 turnover, and 2) Does Sic1 set the Cln threshold? Work on the third technical objective is just underway and will be addressed in a future progress report.

The work described below addresses how cell cycle progression is coordinated with cell growth. It involves the identification of a key in vivo substrate, Sic1, of G1 cyclin dependent kinases, and examines how the interaction and the subsequent phosphorylation of Sic1 coordinate cell cycle progression.

Almost all cells must grow between divisions. This means in order to maintain a homogeneously sized cell population, cells must co-ordinate their growth rate with the division. *In S. cerevisiae* size control occurs at Start where commitment to budding and DNA replication

is dependent upon the attainment of a critical cell size. I proposed a model predicting that for cells to pass Start they must accumulate Cln protein and Cln-Cdc28 kinase activity above a certain threshold. I found that cells absolutely required a critical amount of the Clns to execute Start. Slightly sub-critical levels of Cln are insufficient, and this cannot be circumvented through the accumulation Cln-Cdc28 phosphorylated substrates or through the cumulative effect of these phosphorylations. Furthermore, I showed that Cln accumulation was completely rate limiting for Start. I hypothesized that the critical cell size for division was in fact a "critical Cln threshold." When I found that cell size determines the rate of protein synthesis, it became clear that Clns were not a metric of cell size but rather a gauge of protein synthesis rates. The serum/growth factor dependent step of the restriction point in mammalian cells illustrates the conservation of this mechanism (9).

To understand how cell growth triggers division, I asked whether rapidly and slowly growing cells required the same amount of Cln for Start? In contrast to the B-type cyclins, I have shown that the Clns are constitutively unstable proteins (see section B). Equilibrium levels of unstable proteins are dependent on their rates of synthesis. How then could slowly growing cells with low rates of protein synthesis ever achieve as much Cln as rapidly growing cells? In fact they don't, slowly growing cells express and require much less Cln than rapidly growing cells. Rapidly growing cells have a high "Cln threshold" for Start that falls as their growth rate slows. This explains how cell cycle progression is linked to growth rate, and clarifies how cells rapidly adapt to changing growth rates. At low growth rates, cells divide by lowering their Cln requirements.

I went on to show that the Clb kinase inhibitor, Sic1, largely determines the timing of DNA replication (Manuscript enclosed). I found that Sic1 is a phosphoprotein in vivo, and that Cln-Cdc28 kinase complexes are required for its phosphorylation. Furthermore, Sic1 is lost at Start, and its loss absolutely requires the simultaneous activity of Clns and a ubiquitin conjugating enzyme, Cdc34. Thus, Cln-Cdc28 directly or indirectly phosphorylates Sic1 promoting its degradation via Cdc34. Strikingly, I found that deletion of *SIC1* restored viability to a *cln*-less strain. In the absence of Sic1, Clb-Cdc28 complexes actively initiate S-phase. This result identified a substrate specific for Start and hinted at how Clns drive the initiation of S-phase at Start; thus, Sic1 is a key Cln-Cdc28 substrate, and the only essential function of the Clns is to promote the degradation of Sic1. A battery of recent papers strongly suggests that cell cycle regulated proteolysis may constitute the major mechanism whereby cyclin dependent kinases irreversibly drive cell cycle progression (reviewed in 3).

Finally, I asked how are Start events coordinated? Because Sic1 is an inhibitor of S-phase, I predicted that deletion of *SIC1* might uncouple S-phase from other Start-dependent events. This was true. Wild type and *sic1* mutants bud at identical cell sizes, but *sic1* mutants initiate S phase significantly earlier than wild type cells. Thus, two of the three Start events, budding and DNA replication, are uncoupled and no longer occur simultaneously. Perhaps the other two Start events are coordinated in a similar manner; Cln-Cdc28 phosphorylations may ensure that Start events occur simultaneously and only once per cycle, by driving the degradation of cell cycle inhibitors. Not only does Sic1 appear to have a central role in regulating proliferation in yeast, but it also appears to have a role partially analogous to the RB tumor suppressor gene in humans. In yeast, sic1 mutants rescue the viability of cln-deficient cells (1, 22). This suggests that the only essential function of G1 cyclins may be to remove Sic1.

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Similarly, while cyclin D1 overexpression is associated with nearly half of breast tumors as well as a large percentage of other tumors, it never coincides with loss of Rb function (16).

I am currently investigating how Sic1 levels effect cell cycle progression. I have been able to show that Sic1 protein levels peak prior to Start, and decline rapidly after Start. I have preliminary evidence that this is due to the phosphorylation of Sic1 on multiple sites by Cln-Cdc28 kinase complexes. The exact molecular mechanism of this process has recently been described in very nice detail (23-26). Furthermore it has been nicely established that mutation of consensus phosphorylation sites in Sic1 stabilizes the protein (26). Overexpression of stabilized version of Sic1 results in a G1 cell cycle arrest with cells unable to initiate DNA synthesis (26). I am currently examining the turnover of Sic1 throughout the cell cycle and determining whether Sic1 is extremely unstable between G1 and G2. Finally I am interested in determining whether Cln-Cdc28 complexes are required for Sic1 instability after Start or whether other kinases (e.g. Pcl-Pho85 cyclin dependent kinases) play a role.

In related experiments, I am examining the levels of Sic1 protein at different growth rates. As discussed above, I have shown that Cln levels are highly regulated by growth rate. Since Cln levels are high in asynchronous rapidly growing cells, I postulated that Sic1 levels should also be high. My model predicted that Sic1 levels set the "Cln threshold" for Start. Surprisingly, however, I found that Sic1 levels were very low in rapidly growing asynchronous cells. Furthermore, I found that an extra copy of Sic1 in rapidly growing cells had no effect on the size or timing of S-phase initiation. In contrast, I have shown that an extra copy of Sic1 did effect the levels of Cln-Cdc28 kinase complexes required for S-phase initiation. That is, it greatly delayed the initiation of S-phase in cells with very little Cln-Cdc28 kinase activity. The model I am testing for this apparent contradiction is as follows. Sic1 is stable in the absence of Cln-Cdc28 activity. This occurs only in a window in early G1. This window is very small in rapidly growing cells, and as I have shown (see above), the amount and the rate at which Cln-Cdc28 kinase complexes accumulate in rapidly growing cells is very high. This makes rapidly growing cells relatively insensitive to Sic1 protein levels. The converse is true for slowing growing cells. They have very low levels of Cln-Cdc28 kinase and are therefore, very sensitive to Sic1 levels. I am in process of testing this model. This model predicts that Sic1 acts to prevent cells growing in poor conditions to commit to cell cycle progression until a high rate of protein synthesis to complete the cell cycle.

Clns are highly Unstable activators of Cell cycle progression

A little over 25 years ago, it was first demonstrated that cells require external mitogenic signals to become committed to cell cycle progression (9). This commitment point became known as the "restriction point" in mammalian cells and Start in *S. cerevisiae* (9). It was rapidly well established that progression past Start or the restriction point was dependent upon external mitogenic signals and a high rate of protein synthesis. The removal of growth factors, extracellular nutrients or the addition of protein synthesis inhibitors abruptly arrested cells in G1, thus demonstrating that cell cycle progression is promoted by unstable activator molecules--R proteins. By using short pulses of cyclohexamide in cultures of *S. cerevisiae*, Shilo *et. al.* predicted that these activators had a half-life of about 6 minutes (27). Later, genetic analyses in *S. cerevisiae* revealed the complete dependence of cell cycle progression upon a single cyclin

dependent kinase (Cdk), Cdc28, and the expression of a least one unstable G1 cyclin (Clns). G1 cyclins assumed the role of the unstable activator of cell cycle progression.

While the instability of mitotic B-type cyclins is cell cycle regulated, it was generally accepted that the instability of G1 cyclins is not. A number of recent reports have implicated both Cln-Cdc28 and Clb-Cdc28 complexes as key regulators of cell cycle dependent proteolysis (reviewed in 3). In this context, and because Cln stability had always been measured in asynchronous cultures in the past, it seemed reasonable on the surface to propose that Cln stability could also be cell cycle regulated. Recently, it was reported that G2 cyclins are required for the proteolysis of the G1 cyclins Cln1 and Cln2 and inferred that Clns were stable in pre-Start G1 cells (28). This prediction did not seem to fit with all of the historical evidence that exit from the G1 phase of the cell cycle was dependent upon a unstable molecule. Furthermore, it directly contradicted previous experiments (see section A) indicating that Clns were extremely unstable in G1. Because of the critical role G1 cyclins play in cell cycle regulation, and because the half-life of Cln1/2 had never been directly tested in G1, I set out in collaboration with three other laboratories (Mikes Tyers, Mike Mendenhall, and Curt Wittenberg) to determine if Cln protein is in fact constitutively unstable.

To examine the G1-phase stability of Cln2 directly, we measured the half-life of Cln2 in small G1 phase cells obtained by elutriation. We used strains that contained either WT $CLN2^{HA}$ or a stabilized mutant, $CLN2^{4T3S\text{-}HA}$, both expressed from the $GAL1$ promoter. $Cln2^{4T3S}$ is a multiple point mutant that that lacks seven consensus Cdc28 phosphorylation sites and is 7-fold more stable than wild-type Cln2 in asynchronous cultures (29). The half-life of the mutant and wild type proteins were measured in G1 cells by decay of the Cln2 signal after repression of the $GAL1$ promoter by glucose. Wild type Cln2 had a half-life of 5-10 minutes in the G1 cells. In contrast, the stabilized mutant $Cln2^{4T3S}$ had a longer half life. This experiment shows directly that Cln2 is unstable in G1 phase and suggests that Cln2 instability is required for proper regulation of Start. We also determined that neither tag position nor the epitope sequence altered Cln2 stability.

Furthermore, we compared the ability of untagged $GAL1\text{-}CLN2$ and untagged $GAL1\text{-}CLN2^{4T3S}$ to promote Start. Small G1 cells were obtained by elutriation, and then the $GAL1$ promoter was turned on slightly with a low concentration of galactose in a semi-repressing medium. G1 expression of $Cln2^{4T3S}$ accelerated Start and reduced critical cell size compared to expression of wild-type Cln2. The differential ability of stabilized Cln2 to promote Start again suggests that wild type Cln2 is unstable in G1 cells.

Because the recent suggestion that the Clb mitotic cyclins are necessary for Cln degradation is inconsistent with our finding that Cln2 is unstable in G1 cells (28), where Clb cyclins are absent (30, 31), we measured Cln2 stability under three different conditions that severely reduce or eliminate Clb-Cdc28 activity. Cells lacking Clb1 through Clb4 arrest in G2 phase, while cells lacking Clb1 through Clb6 arrest in G1 phase (30-32). Cells that overexpress the Clb-Cdc28 inhibitor Sic1 also accumulate in G1 phase (26). When Cln stability was measured in a $clb1\text{-}4^{ts}$ strain by repression of a $GAL1\text{-}CLN2^{HA}$ or a $GAL1\text{-}CLN1^{HA}$ construct, Cln was no more stable than in wild type cells. Cln2 was also unstable in cells arrested in G1 by overexpression of a stabilized version of Sic1.

Finally, we re-examined the role of the ubiquitin conjugating enzyme Cdc34 in the turnover of Cln2. It has recently been argued that Cdc34 promotes Cln degradation indirectly by

promoting degradation of Sic1, thereby activating Clb kinases (28). If this were true, then *cdc4* mutants should also be defective in Cln2 turnover, since Sic1 accumulates in *cdc4* mutants just as it is in *cdc34* mutants. However, Cln2 degradation was defective only in the *cdc34* mutant, despite the fact that Sic1 accumulated in both the *cdc34* mutant and the *cdc4* mutant. This indicates that that Cln stabilization in *cdc34* mutants is not an indirect effect of Sic1 accumulation and loss of Clb activity.

A model in which G1 cyclins are stable in G1 phase and then catastrophically destroyed as cells pass Start, is attractive. However, our data rule out this model and support a model in which Cln proteins are constitutively unstable. The instability of the Cln proteins probably derives from Cdc28 dependent autophosphorylation of the Cln subunit, which targets the Cln for degradation (29, 33, 34). The intrinsic instability of the Cln proteins is advantageous for the cell because it allows almost instantaneous responses to changes in the rate of protein synthesis and *CLN* transcription, both of which are key determinants of whether or not cell division is appropriate (35, 36). The continuous requirements for growth factors and protein synthesis in G1 phase progression of mammalian cells, and the phosphorylation dependent instability of cyclin D and cyclin E, suggests that similar control of G1 cyclin activity operates in mammalian cells (37-39).

In conclusion, we report that Clns are highly unstable in all phases of the cell cycle, that Clb-Cdc28 complexes have little or no role in regulating the stability of Clns, and that Clns, in fact, do appear to be the rate-limiting unstable activators of Start predicted by Shilo et al. nearly 20 years ago (27). This is in agreement with recent reports identifying mammalian G1 cyclins as a potential candidate for the R-protein in humans. (40).

C. Future Work

How does Sic1 or other key substrate(s) set the Cln threshold for Start?

I have shown that Cln thresholds exist (Schneider *et al.*, in preparation); if they are set by Sic1, then I expect Sic1 abundance to change as a function of growth rate, just as the threshold does. By examining the abundance and half-life of Sic1 as a function of cell cycle position, I will determine if Sic1 is a generally unstable protein whose instability is greatly increased in late G1 due to Cln accumulation. By testing Sic1 mutants that cannot be phosphorylated or mutants that mimic phosphorylation, I will determine the effect of phosphorylation on Sic1 stability.

Sic1 has no known sequence homologues. Thus, I will confirm if Sic1 is indeed an RB analog, or if there are in fact human Sic1 sequence homologues, and whether it shares other similarities, *i.e.*, does Sic1 interact with the transcription factors Swi4/Swi6/Mbp1 like RB interacts with E2F. Maybe, like RB, it is the hypo-phosphorylated form of Sic1 that inhibits cell cycle progression, and so I will examine the role of known phosphotases on the phosphorylation state of Sic1. The aim of this research is to rigorously determine the role of Sic1 in cell cycle progression.

In addition, I propose to screen for other mutants that lower the "Cln threshold", but not to zero. Desired mutants would still need Clns, but at lower levels than wild-type cells require. Preliminary experiments have yielded encouraging results, and I am in the process of characterizing these mutants.

What mechanism couples and coordinates initiation of budding to Start?

Cell cycle regulated proteolysis appears to be the major mechanism whereby cyclin-Cdk complexes irreversibly drive cell cycle progression. Sic1 "couples" S-phase to Start; perhaps other "coupling proteins" exist. I hypothesize that Cln-Cdc28 complexes target other substrates for degradation, and I would like to identify a budding inhibitor or another Cln-Cdc28 substrate that links budding to Start. Another cyclin-Cdk complex, Pcl-Pho85, appears to have a role in bud initiation, and the Pho81 gene is a known Pho85 kinase inhibitor. Perhaps Pho81 is the budding equivalent of Sic1. I will test the effects of overexpression of *PHO81* or Pcl-Pho85 kinase complexes, or the deletion of *pho81* on the timing of budding. In addition, I will screen for mutants that can bud in the absence of Cln-Cdc28 activity. Alternatively, it could be that Cln-Cdc28 activates a protein, which promotes budding. I will clone activators of budding by identifying genes that on high copy plasmids induce budding in the absence of Clns.

Conclusion

The regulation of cell cycle progression is a basic biological problem. However, very little is known about the targets of Cdk phosphorylations, and how commitment to cell division is controlled. I have identified Sic1, a potential analog to the RB tumor suppressor gene, as a key Cdk substrate whose phosphorylation has a central role in regulating proliferation. I have shown that Clns like cyclin D/E in mammalian cells are highly unstable rate limiting activators of cell cycle progression. This makes them excellent candidates for the proposed R-proteins in yeast. I have shown that Cln levels and Sic1 levels are intricately related, and that it is likely that Sic1 acts to restrain cell cycle progression under poor growth conditions. The relationship between cell size, cell growth rate, and Cln activity is an ongoing puzzle, and I will continue to investigate these inter-relationships. A better working model of how Sic1 controls cell cycle progression may go a long way towards understanding how cell cycle control effects the onset of cancer

References

1. Schneider, B.L., Yang, Q.-H., and Futcher, A.B. Linkage of replication to Start by the CDK inhibitor Sic1. *Science* 272, 560-562. (1996).

2. Schneider B.L., Patton, E.E., Lanker, S. Mendenhall, M.D., Wittenberg, C., Futcher, B. and Tyers, M. Yeast G1 cyclins are unstable in G1 phase. *Nature* (in press).

3. Hoyt, M.A. Eliminating all obstacles: Regulated proteolysis in the eukaryotic cell cycle. *Cell* 91, 149-151. (1997).

4. Futcher, B. Cyclins and the Wiring of the Yeast Cell Cycle. *Yeast* 12, 1635-1646, (1996).

5.   Pringle, J.R., and Hartwell, L.H. The *Saccharomyces cerevisiae* cell cycle. The Molecular Biology of the Yeast Saccharomyces, (eds. J.D. Strathern, E.W. Jones, and J.R. Broach. (1981).

6.   Rossow, P.W., Riddle, V.G. & Pardee, A.B. Synthesis of labile, serum-dependent protein in early G1 controls animal cell growth. *Proc Natl Acad Sci U S A* 76, 4446-50 (1979).

7.   Pardee, A.B. G1 events and regulation of cell proliferation. *Science* 246, 603 (1989).

8.   Hartwell, L.H. *Saccharomyces cerevisiae* cell cycle. *Bacteriology Rev.*, 38, 164 (1974).

9.   Zetterberg, A., Larsson O., and Wiman, K.G. What is the restriction point? *Current Biology* 7, 835-842 (1995).

10.   Reed, S.I. The role of p34 kinases in G1 to S-phase transition. *Annu. Rev. Cell Biol.* 8, 529 (1992).

11.   K. Nasmyth. Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.* 5, 166 (1993).

12.   Schmidt, E.V. Happenstance, circumstance or enemy action: cyclin D1 in breast, eye and brain. *Bioessays* 18, 6-8 (1996).

13.   Xiong, Y., Connolly, T., Futcher, B., and Beach, D. Human D-type cyclin. *Cell* 65, 691-699. (1991).

14.   Weinstat-Saslow, D., Merino, M.J., Manrow, R.E., Lawrence, J.A., Bluth, R.F., Wittenbel, K.D., Simpson J.F., Page, D.L., and Steeg, P.S. Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. *Nat. Med.* 1, 1257-1260. (1995).

15.   Zuckerberg, L.R., Yang, W.I., Gadd, M., Thor, A.D., Koerner, F.C., Schmidt, E.V., and Arnold, A. Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. *Mod. Pathol.*, 8, 560-567. (1995).

16.   Sherr, C.J. D-type cyclins. *Trends-Biochem-Sci.*, 20, 187 (1995).

17.   M. D. Mendenhall, C. A. Jones, S. I. Reed. Dual regulation of the yeast CDC28-p40 protein complex: cell cycle, pheromone and nutrient limitation effects. *Cell* 50, 927 (1987).

18.   Nugroho, T.T., and Mendenhall, M. D. An inhibitor of yeast cyclin-dependent protein kinase plays an important role in ensuring the genomic integrity of daughter cells. *Mol. Cell Biol.* 14, 3320 (1994).

19. M. D. Mendenhall. An inhibitor of p34^{CDC28} protein kinase activity from *Saccharomyces cerevisiae. Science* 259, 216 (1993).

20. E. Schwob, T. Bohm, M. D. Mendenhall, K. Nasmyth. The B-type cyclin kinase inhibitor p40^{sic1} controls the G1 to S transition in *S. cerevisiae. Cell* 79, 233 (1994).

21. Donovan, J.D., Toyn, J.H., Johnson, A.L., and Johnston, L.H., P40^{SDB25}, a putative CDK inhibitor, as a role in M/G1 transition in *Saccharomyces cerevisiae. Genes Dev.*, 8, 1640 (1994).

22. Tyers, M. The Cyclin dependent kinase inhibitor p40SIC1 imposes the requirement for CLN G1 cyclin function at Start. *Proc. Natl. Acad. Sci. USA* 93, 7772-7776.

23. Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. & Harper, J.W. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* 91, 209-19 (1997).

24. Feldman, R.M., Correll, C.C., Kaplan, K.B. & Deshaies, R.J. A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* 91, 221-30 (1997).

25. Patton, E.E., *et al.* Cdc53 is a scaffold protein for multiple Cdc34/Skp1/F-box protein complexes that regulate cell division and methionine biosynthesis in yeast. *Genes Dev* 12, 692-705 (1998).

26. Verma, R., *et al.* Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* 278, 455-60 (1997).

27. Shilo, B., Riddle, V.G., and Pardee, A.B. Protein turnover and cell cycle initiation in yeast. *Exp Cell Res.* 123, 221-227. (1979).

28. Blondel, M. & Mann, C. G2 cyclins are required for the degradation of G1 cyclins in yeast. *Nature* 384, 279-82 (1996).

29. Lanker, S., Valdivieso, M.H. & Wittenberg, C. Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science* 271, 1597-1601 (1996).

30. Amon, A., Irniger, S. & Nasmyth, K. Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell* 77, 1037-1050 (1994).

31. Amon, A., Tyers, M., Futcher, B. & Nasmyth, K. Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell* 74, 993-1007 (1993).

32. Schwob, E., Bohm, T., Mendenhall, M.D. & Nasmyth, K. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. *Cell* 79, 233-44 (1994).

33. Willems, A.R., *et al.* Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway. *Cell* 86, 453-63 (1996).

34. Yaglom, J., *et al.* p34Cdc28-mediated control of Cln3 cyclin degradation. *Mol Cell Biol* 15, 731-741 (1995).

35. Ko, H.A. & Moore, S.A. Kinetic characterization of a prestart cell division control step in yeast. Implications for the mechanism of alpha-factor-induced division arrest. *J Biol Chem* 265, 21652-63 (1990).

36. Zetterberg, A. & Larsson, O. Coordination between cell growth and cell cycle transit in animal cells. *Cold Spring Harb Symp Quant Biol* 56, 137-47 (1991).

37. Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M. & Roberts, J.M. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev* 10, 1979-90 (1996).

38. Diehl, J.A., Zindy, F. & Sherr, C.J. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* 11, 957-72 (1997).

39. Won, K.A. & Reed, S.I. Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *Embo J* 15, 4182-93 (1996).

40. Dou, Q-P., Levin, A.H., Zhao, S., and Pardee, A. B. Cyclin E and cyclin A as candidates for the restriction point protein. *Cancer Res.* 53, 1493-1497 (1993).

Appendices:

Curriculum vitae

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Education

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Ph.D. in Microbiology and Immunology
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1981-1986 University of Washington
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Honors, Awards, and Affiliations:

1997- Army Breast Cancer Fellowship

1996-1997 NIH NSRA Fellowship

1994-1995 Long Island Biological Association Fellowship

1991- American Association for Cancer Research member

1991-1992 Outstanding graduate student, Collegiate Institute for Leadership

1990-1993 NIH Predoctoral Cancer Biology Training grant recipient, University of Arizona

1987-1988 Graduate Recruitment Fellowship, University of Arizona

Professional Experience:

1996-present   Directed undergraduate research.

1990-1992   ad hoc referee for Carcinogenesis, Molecular Carcinogenesis, and Cancer Research.

1987-1988   Directed undergraduate research.

Publications:

Schneider B.L., Patton, E.E., Lanker, S. Mendenhall, M.D., Wittenberg, C., Futcher, B. and Tyers, M. (1998) Yeast G1 cyclins are unstable in G1 phase. *Nature* (in press).

Schneider, B.L., Yang, Q.-H., and Futcher B. (1996) Linkage of replication to Start by the Cdk inhibitor Sic1. *Science* 272, 560-562.

Schneider B.L. , Steiner, B., Seufert, W., and Futcher A.B. (1996) pMPY-ZAP: A reusable PCR-directed gene disruption cassette for *S. cerevisiae. Yeast* 12:129-134.

Schneider B.L. , Seufert, W., Steiner, B., Yang, Q.H., and Futcher A.B. (1995) Use of polymerase chain reaction epitope tagging for protein tagging in *Saccharomyces cerevisiae. Yeast* 11:1265-1274.

Schneider B.L. , Kulesz-Martin, M.F., and Bowden G.T. (1995) Induced differentiation and tumorigenic suppression in murine somatic cell hybrids. *Mol. Carcinog.* 13:6-14.

Andrews, K.L, **Schneider, B.L.**, and Bowden, G.T. (1994) PCR-based rescue of cDNAs from functionally transformed cells. *Methods in Molecular and Cellular Biology* 4:249-256.

Bowden, G.T., **Schneider, B.L.**, Domann, R., and Kulesz-Martin, M.F. (1994) Oncogene activation and tumor suppressor gene inactivation during multistage mouse skin carcinogenesis. *Cancer Research* 54:1882s-1885s.

Schneider B.L., Bowden G.T., Sutter C., Schweizer J., Han K.-A., and Kulesz-Martin M.F. (1993) 7,12-dimethylbenz[*a*]anthracene-induced mouse keratinocyte malignant transformation independent of Harvey *ras* activation. *Journal of Investigative Dermatology* 101:595-599.

Schneider B.L. and Bowden G.T. (1992) Selective pressures and *ras* activation in carcinogenesis. *Mol. Carcinog.* 6:1-4.

Manuscripts in Preparation:

Schneider, B.L., Volpe, T., Tokiwa, G.T., and Futcher, A.B. The critical level of G1 cyclin required for Start varies dramatically with growth rate (in preparation).

Nash, R.N., Volpe, T., **Schneider, B.L.**, and Futcher, A.B. Cloning and characterization of *WHI3*, a new size control gene in *S. cerevisiae*. (in preparation).

Abstracts:

Volpe, T., Nash, R.N., **Schneider, B.L.**, and Futcher, A.B. Characterization of *WHI3*, a new size control gene in *S. cerevisiae*. 1997 Yeast Biology Meeting at Cold Spring Harbor Laboratory.

Futcher, B., Yang, Q.-H., Sherlock, G., Marshak, D. and **Schneider, B.** SIC1 and other substrates of CDK kinases. Dept. of Defense Breast Cancer Research Program Meeting: Era of Hope; Maryland, 1997.

Sherlock, G., **Schneider B**. and Futcher , B. Epitope tagging of yeast proteins using PCR approaches. FASEB yeast chromosome structure, replication and segregation meeting, Colorado, 1996.

Schneider, B.L., Yang, Q.-H.., and Futcher A. The Cdk inhibitor Sic1 links S-phase to Start. 1996 Yeast Genetics and Molecular Biology Meeting.

Schneider, B.L., and Futcher A. How to PET your favorite gene. 1996 Yeast Genetics and Molecular Biology Meeting.

Schneider, B.L., Yang, Q.-H.., and Futcher A. The Cdk inhibitor Sic1 helps set the Cln threshold for Start. 1996 FASEB Yeast DNA replication meeting.

Schneider, B.L., Volpe, T., and Futcher A. Size Matters: Cln thresholds link cell size and growth rate at Start. 1996 Yeast Biology Meeting at Cold Spring Harbor Laboratory.

Futcher, B., Yang, Q.-H., Tokiwa, G., and **Schneider, B.L.** Divide or Bust: Critical Cln thresholds for Start in budding yeast. 1996 Keystone Symposium.

Schneider, B.L., Tokiwa, G.T., and Futcher A. Divide or Bust: Critical Cln thresholds for Start in budding yeast. 1995 Yeast Biology Meeting at Cold Spring Harbor Laboratory.

Schneider B.L. and Bowden G.T. A PCR-based biotin-avidin subtraction technique for cloning tumor suppressor genes, 1992 Oncogene meeting.

Schneider B.L. and Bowden G.T. Differential gene expression of keratins K6 and K18 as markers of tumor progression *in vitro*, 1992 AACR meeting.

Schneider B.L., Kulesz-Martin M.F., and Bowden G.T. An epithelial cell model to chronicle the loss of tumor suppressor activity during multi-stage carcinogenesis, 1991 Oncogene meeting.

Han K.-A., **Schneider B.L.**, and Kulesz-Martin M.F. Abnormal expression of cellular VL30 and p53 genes and absence of *ras*-like transforming activity in a murine keratinocyte/tumorigenesis model, 1989 Gordon Conference.

Schneider B.L., Kulesz-Martin M.F., and Bowden G.T. The use of somatic cell hybrids to study the regulation of the tumorigenic phenotype in an in vitro model of multi-stage carcinogenesis,. 1989 Summer FASEB Conference.

Gaur L.K., **Schneider B.L.**, Valentine M., Nelson K.A., Pesando J., Parham P., and Clark E.A. Polymorphic class II antigens in macaques detected by mouse anti-HLA monoclonal antibodies, 1987.